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14. ABSTRACT
Animal research suggests that reactivation (retrieval) of a consolidated memory can return it to a labile state from which it must be restabilized in order to persist. This stabilization process has been termed "reconsolidation", and various behavioral and pharmacological interventions have been found to modify or block it. The aim of this project is to create an experimental assay in the form of an optimal Pavlovian differential fear-conditioning paradigm, within which the relative strengths of various pharmacological and behavioral, reconsolidation-blocking interventions can be tested. Thus far, we have completed the testing for the pharmacological intervention group and are continuing the recruitment for the behavioral intervention group. Data from the pharmacological group demonstrate that participants show differential conditioning learning on Day 1, supporting the validity of our modified fear-conditioning paradigm. Results suggest that propranolol administration at the time of memory reactivation does not decrease the fear memory, as indexed by skin conductance, when assessing renewal and reinstatement. When looking at spontaneous recovery at Day 30, results suggest the emergence of a latent effect, which remains to be confirmed and understood better. The addition of a placebo control group is needed to make a conclusive statement about the efficacy of propranolol.

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1. INTRODUCTION

Background: Animal research suggests that reactivation (retrieval) of a consolidated memory can return it to a labile state from which it must be restabilized in order to persist. This stabilization process has been termed "reconsolidation," and various pharmacological and non-pharmacological interventions can block it. This ability offers novel therapeutic possibilities for PTSD. To date, few human studies have been conducted on the mechanism of memory reconsolidation blockade; some of them have yielded positive results but their clinical relevance to the problem of PTSD remains very limited. Two recent studies performed in healthy human subjects have addressed the question of memory reconsolidation blockade using fear-conditioning paradigms, which are highly relevant to PTSD. Both studies demonstrated that fear memory could be eliminated via the mechanism of reconsolidation. The first study (Kindt et al. 2009) demonstrated the phenomenon using a beta-blocker, propranolol, whereas the other study (Schiller et al. 2010) obtained similar results using a non-pharmacological/behavioral intervention that combines an extinction protocol within a reconsolidation paradigm (delayed extinction). Given that both studies quickly and completely abolished fear responses, this floor effect prevents us from using their study design in order to test the relative strengths of various reconsolidation-blocking (or memory updating) interventions. In other words, if propranolol or delayed extinction totally eliminates the conditioned fear response, no other intervention could be found to be superior.

Goals: The specific aim of the present project is to create an experimental assay in the form of an optimal Pavlovian differential fear conditioning paradigm within which the relative strengths of various novel behavioral and pharmacological reconsolidation-based interventions can be compared. In order to accomplish this, we designed a new experimental protocol that is free of floor effects. Specifically, we are testing the following modifications to existing experimental designs: 1) use of a more highly "prepared" (i.e., danger-signaling) conditioned stimulus (CS); 2) recruitment of more sensitive subjects; 3) selection of only subjects who acquire strong conditioned responses (CRs) during conditioning for further participation, and 4) use of additional probes for the presence of the latent CR, viz., renewal and savings in addition to spontaneous recovery and reinstatement.

General procedure: The protocol consists of four distinct visits taken place over the course of a month. On Day 1 (habituation and acquisition), subjects view video clips of three rooms (contexts) different in color and content, presented on a 42" high definition television. The stimuli are three different videos of tarantulas, one presented in the context of each room. Two of the three tarantulas serve as the two CS+s and the third as the CS-. Each CS+ presentation is sometimes followed by shock (i.e. reinforced); the CS- is never be followed by shock. During this first session, each CS is presented twice in an unreinforced manner (habituation phase). Next, during the acquisition phase, there are 8 presentations of each CS, with 5 of each CS+ presentations followed by shock (i.e. 62.5% reinforcement). The acquisition phase takes place in Context A. On Day 2 (intervention), subjects are assigned to one of the two conditions: pharmacological or behavioral intervention. For the former, participants are given 40 mg of propranolol (oral administration) that is followed 90 minutes later by a single, unreinforced presentation of one of the two CS+, designated the reactivated CS+ (CS+R). For the behavioral intervention, participants are exposed to a single, unreinforced presentation of the CS+R without

receiving a pill, followed 10 minutes later by 10 further unreinforced CS+R presentations, 11 unreinforced presentations of the remaining CS+ (designated the CS+ with no intervention – CS+N) and 11 CS- presentations. All presentations related to Day 2 take place in context B. Day 3 is divided into two different components, all taking place in context A. First, each CS (CS+R, CS+N and CS-) is presented twice in an unreinforced manner in order to assess renewal. This is followed by the presentation of 3 shocks alone and 8 further unreinforced presentations of each CS (CS+R, CS+N and CS-) in order to evaluate reinstatement. Finally, on Day 30, all presentations take place in a new context, namely context C. In order to evaluate spontaneous recovery, 8 unreinforced presentations of each CS (CS+R, CS+N, CS-) are presented first. This is followed by 8 presentations of all CSs, with the two CS+s being reinforced 62.5% of the time. This second acquisition phase allows us to examine savings during re-acquisition.

Importantly, although the same spider is always used for the CS-, two different task versions were designed so that the two remaining spiders are alternated regarding whether they serve as CS+R or CS+N.

2. BODY

Subject recruitment: At the end of the 01 year, we had recruited a total of 32 subjects, resulting in 6 drop outs and 26 completions. At of the end of the 02 year, we have recruited a total of 85 subjects. Among them, 46 have completed all four testing sessions, 12 completed the initial three visits but were subsequently lost to follow-up, and 1 has dropped out for reasons unrelated to the experimental procedures (e.g. participant reported being too busy). The remaining 26 subjects were screened out of the experiment due to: a) lack of a skin conductance response during their first session, b) failure to demonstrate differential conditioning in their first session, or c) equipment failure. Importantly, our recruitment goal of two participants per week, as reported in the Statement of Work, has not been met. However, it is however important to mention that submission for the addition of a placebo control group (see below in body section) as well as a failure of the laboratory computer hard drive (which has been fixed) have recently slowed us down and have prevented us from meeting our full recruitment goal. During this period, we have nonetheless screened 26 more participants, 13 of whom have met study criteria and are ready to be scheduled in the coming weeks.

Of the 85 participants who have been recruited, 50 have been assigned to the pharmacological intervention (44 of them generated data that were used in the analyses below) whereas the remaining 35 have been assigned to the non-drug/behavioral intervention (only 9 of which have demonstrated adequate conditioning of a fear response to both the CS+R and CS+N, a study requirement for continued participation).

Results: For the pharmacological intervention group, we performed statistical analyses for the four different testing sessions using all data that were available to us (see Figure 1). In order to determine whether participants showed differential conditioning on Day 1, two 2-way repeated measures ANOVAs were performed on the acquisition trials, with Stimulus (CS+, CS-) and Trials (8) as main effects. Separate ANOVAs were used to compare the CS+R (to-be-reactivated CS+) with the corresponding CS- trials and the CS+N (not-to-be-reactivated CS+) with its corresponding CS- trials. For the analysis comparing the CS+R trials (dark blue line) to the

respective CS- trials (yellow line), a main effect of Stimulus was observed that demonstrated larger skin conductance responses to the CS+R trials, $F(1, 43) = 25.89$, $p < 0.001$. A similar pattern was observed when comparing the CS+N trials (pink line) with its respective CS- trials (yellow line), $F(1, 43) = 15.76$, $p < 0.001$.

In order to investigate the impact of propranolol administration at the time of memory reactivation on renewal, a two-way repeated measures ANOVA with Stimulus (CS+R, CS+N and CS-) and Trials (2) was conducted on the first six trials of Day 3. The analysis yielded a main effect of Stimulus, $F(1.94, 67.94) = 13.48$, $p < 0.001$. Follow-up pair-wise comparisons showed that both CS+s (reactivated and non-reactivated) did not differ from each other ($p = 0.797$), but differed significantly from the CS-, p's ≤ 0.001.

In order to investigate the impact on reinstatement, a repeated measures ANOVA was conducted with Stimulus (3) and Trials (2) on the six trials following the reinstatement shocks (presented as yellow lightning bolts on the graph). The ANOVA yielded a main effect of Stimulus, $F(1.86, 65.18) = 4.70$, $p = 0.014$. Follow-up pair-wise comparisons showed that skin conductance responses were marginally larger to the CS+R, compared to the CS-, $p = 0.081$ and that the skin conductance responses to the CS+N were significantly larger than those to the CS-, $p = 0.01$.

At Day 30, repeated measures ANOVA was used to examine the first six trials (two trials per stimulus) in order to assess spontaneous recovery of the fear memory. A main effect of Stimulus, $F(1.631, 40.780) = 6.488$, $p = 0.006$ as well as a main effect of Trials, $F(1, 25) = 17.52$, $p < 0.001$ was found. Pair-wise comparisons revealed that skin conductance responses to the CS+R were not significantly larger than those to the CS- ($p = 0.234$) and were significantly smaller than those to the CS+N, $p = 0.048$. Moreover, skin conductance responses to the CS+N were significantly larger than skin conductance responses to the CS-, $p = 0.001$. Extinction learning was assessed by a repeated measures ANOVA that included Stimulus (3) and Trials (10) as main effects. Significant main effects for both Stimulus, $F(1.687, 42.171) = 3.26$, $p = 0.056$, and Trials, $F(4.611, 115.274) = 12.65$, $p < 0.001$, emerged. Moreover, the interaction between these two factors was significant, $F(5.892, 147.298) = 2.18$, $p = 0.05$. Upon decomposing the interaction, it was only found that skin conductance response magnitude showed a significant decline over trials (F's ≥ 2.68, p's ≤ 0.031. When comparing the CSs using pair-wise comparisons, only CS+N yielded significantly larger skin conductance responses than CS-, $p = 0.015$. Finally, when examining the re-acquisition trials, the ANOVA comparing the CS+R to the corresponding CS- trials revealed a main effect of Trials, $F(3.189, 94.04) = 3.07$, $p = 0.21$, and a main effect of Stimulus, $F(1, 24) = 6.49$, $p = 0.018$, where the CS+ elicited larger skin conductance responses than the CS-. The ANOVA comparing the CS+N to the corresponding CS- trials also revealed a main effect of Trials, $F(4.63, 110.99) = 2.78$, $p = 0.024$, and a main effect of Stimulus, $F(1, 24) = 3.92$, $p = 0.004$, with the CS+N eliciting larger skin conductance reponses the CS-. The Stimulus x Time interaction was marginally significant, $F(4.012, 96.29) = 2.36$, $p = 0.059$. Finally, the ANOVA comparing skin conductance responses to the CS+R with those to the CS+N only revealed a significant effect of Trials, $F(4.232, 101.566) = 4.714$, $p = 0.001$, suggesting that skin conductance responses to the CS+R and CS+N did not differ from each other.

It is important to note that a primary goal of this research has been to create a paradigm that allowed for testing of the impact of different interventions (pharmacological and behavioral) on memory reconsolidation, while avoiding the floor effects that has characterized recent

reconsolidation studies. In keeping with our goal of eliminating the floor effect observed in previous studies, we reexamined our data selecting only participants who showed strong differential conditioning to the respective CS+R *and* CS+N on Day 1. This resulted in an exclusion of 26 participants who did not show clear differential conditioning to both CS+s. Data for the subset of participants who showed strong differential fear conditioning to the CS+R and CS+N (n=18) were analyzed separately (see Figure 2) and yielded very similar results to what is reported above.

In fact, on Day 1, when comparing the CS+R with the CS-, a main effect of Stimulus, $F(1, 17) = 59.41$, $p < 0.001$, as well as a main effect of Trials, $F(7, 119) = 4.07$, $p < 0.001$, were observed. A main effect of Stimulus was also observed when comparing the CS+N with the CS-, $F(1, 17) = 36.58$, $p < 0.01$. Moreover, there was a significant interaction between Stimulus and Trials, $F(7, 119) = 2.44$, $p = 0.02$. Both CS+s produced larger skin conductance responses than the respective CS- trials and the skin conductance responses to the CS+R and CS+N did not differ from each other ($p = 0.36$).

When examining renewal on Day 3 in the reduced sample, a main effect of Stimulus, $F(1, 17) = 16.16$, $p < 0.001$, as well as a significant Stimulus x Trials interaction, $F(1, 17) = 4.6$, $p = 0.04$, were found when comparing skin conductance responses to the CS+R with those to the CS-. Comparison of the skin conductance responses to the CS+N with those to the CS- produced significant Stimulus and Trials main effects, F's $(1, 17) \geq 5.21$, p's ≤ 0.04. Importantly, both CS+s did not differ significantly from each other, $p = 0.37$. An examination of skin conductance during reinstatement produced a significant Stimulus main effect, $F(1, 17) = 5.43$, $p = 0.03$ with the CS+R producing larger skin conductance responses than the CS-, as was also found when comparing CS+N trials with its respective CS- trials, $F(1, 17) = 3.41$, $p = 0.008$. Skin conductance response magnitude did not differ between the CS+R and CS+N ($p = 0.66$).

Analysis of variance (Stimulus (3), Trials (2)) for the Day 30 reduced-sample data that examined spontaneous recovery yielded a significant interaction, $F(1.742, 20.907) = 3.69$, $p = 0.048$. The decomposition of this interaction revealed that skin conductance responses to CS+R showed a significant decrease from Trial 1 to 2 ($p < 0.05$), whereas this was not the case for the CS+N and CS-. Analysis of extinction (Trials 1-10) only yielded a main effect of Trials, $F(3.171, 38.052) = 9.55$, $p < 0.001$. For the re-acquisition phase, the ANOVA comparing skin conductance responses to the CS+R with those to the corresponding CS- trials revealed a main effect of Stimulus, $F(1, 11) = 11.59$, $p = 0.006$, a main effect of Trials, $F(3.238, 35.623) = 3.05$, $p = 0.038$, as well as a significant interaction, $F(3.576, 39.336) = 3.19$, $p = 0.027$. Overall, the CS+R produced larger skin conductance responses than did the CS-. The ANOVA comparing responses to CS+N trials with its corresponding CS- trials only revealed a Stimulus main effect, $F(1, 12) = 7.76$, $p = 0.016$, whereby the CS+N elicited larger skin conductance responses than did the CS-. Finally, the ANOVA contrasting CS+R and CS+N trials revealed a significant Trials main effect, $F(3.675, 40.42) = 3.49$, $p = 0.018$. This suggests that overall, skin conductance responses to the CS+R were comparable in magnitude to responses to the CS+N.

Discussion

Taken together, results from the full and restricted samples support the efficacy of our sampling strategy and conditioning task for producing robust differential conditioning. The results we obtained on Day 3 suggest that a 40mg dose of propranolol is not effective in blocking

reconsolidation of the memory associated with a conditioned fear response, as indexed by skin conductance reactivity. However, when examining on Day 30 the spontaneous recovery and re-extinction of the previously established fear response, and taking into account the data from all participants, results appear to suggest that skin conductance response magnitude to the CS+R did not differ significantly from the CS-, whereas a difference was observed between the CS+N and CS-, suggesting that the propranolol may have interfered with memory re-consolidation over the longer term. However, it is important to note that these effects are not present when looking at the subset of individuals who showed initially strong differential conditioning on Day 1, but only 13 individuals from this subset completed Day 30 (as opposed to 25 individuals when taking all participants into account). How best to interpret the Day 30 finding is not clear at this point. If the finding proves to be robust, a mechanism that can explain the latent reduction in conditioned fear will need to be considered.

Although the present work raises some doubt as to whether a 40mg dose of propranolol can measurably reduce reconsolidation of a conditioned fear response, several points should be considered. First, previous studies have reported the ability of propranolol to eliminate the fear memory trace completely, thus creating floor effects. Our study, however, was specifically designed to avoid these floor effects and to allow exploring and comparing different potential interventions for blocking memory reconsolidation. It is quite possible that our modified paradigm created memory traces that were stronger and not as readily destabilized as those created in previous studies. In order to conclude that propranolol partially blocked memory reconsolidation, one would expect the CS+R to elicit significantly reduced skin conductance responses compared to the CS+N, both at Day 3 and Day 30. The present findings suggest that skin conductance reactivity to these stimuli did not differ significantly and that the CS+s produced comparably strong differential conditioning (i.e., when compared to the CS-); although, the possibility of a latent effect at Day 30 does exist.

It is possible that the single presentation of the CS+R on Day 2 sensitized subjects to the CS+R, but that evidence of this sensitization was blocked by the propranolol, resulting in comparably larger skin conductance responses to the CS+R and CS+N on Day 3. Thus, one cannot rule out the possibility that the comparability of responding to the CS+R and CS+N reflected an attenuation of a sensitized response to the CS+R. For this reason, we have decided to add a placebo group to the current study. This will allow us to make a stronger statement about the effects of propranolol on memory reconsolidation blockade in the context of our modified fear-conditioning paradigm. An amendment for this request was sent to our local IRB on December 4th 2012 and is currently under consideration/review by HRPO.

3. KEY RESEARCH ACCOMPLISHMENTS

Based on our recently submitted revised statement of work, we have met the proposed objectives. Here are the main steps that have been accomplished over the last year:

- testing completed for the group assigned to the pharmacological blockade of memory reconsolidation using the newly developed conditioning paradigm

- finalizing the statistical analyses of data from the group assigned to the pharmacological blockade of memory reconsolidation

- submission and acceptance of a Poster to be presented at the Society of Biological Psychiatry meeting in May 2013

- recruitment and testing of 35 participants assigned to the behavioral intervention group

- protocol and IRB amendment to add a placebo group to the current study

4. REPORTABLE OUTCOMES

The results obtained and analyzed from the pharmacological intervention group have been presented at a group meeting in Montreal in January 2013. This is a small group of researchers, who are all experts in memory reconsolidation and who meet twice a year to discuss recent research developments and inform the group of the progress of their own memory reconsolidation research.

The findings to date will be presented and discussed via Poster Session, in May 2013 at the annual Society of Biological Psychiatry Meeting in San Francisco, California.

5. CONCLUSION

The data and findings obtained to date suggest that our goal of creating a modified fear-conditioning paradigm that is free from intervention floor effects associated with blockade of memory reconsolidation has been achieved. While the paradigm has been successful, results obtained on Day 3 in the pharmacological intervention group suggest that 40mg of propranolol does not block reconsolidation of the fear memory. Our examination of skin conductance reactivity representing the fear memory at Day 30 is somewhat more promising and suggests that a latent effect may have emerged. However, when considering the reduced subset of individuals who showed good differential conditioning on Day 1, this effect was not present. For now, we cannot rule out the possibility that a lack of statistical power is responsible for the lost effect. In order to better understand the present findings, and whether propranolol may be having an effect that is hidden or reduced by sensitization resulting from the reactivation trial on Day 2, we believe it is important to run a placebo comparison group. We will therefore concentrate our efforts in the coming months for recruiting and testing a placebo group as well as pursuing the testing of the behavioral intervention group and an additional pharmacological intervention.

6. REFERENCES

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Schiller D, Monfils MH, Raio CM, Johnson DC, Ledoux JE, Phelps EA. Preventing the return of fear in humans using reconsolidation update mechanisms. *Nature* 2010;463:49-53.

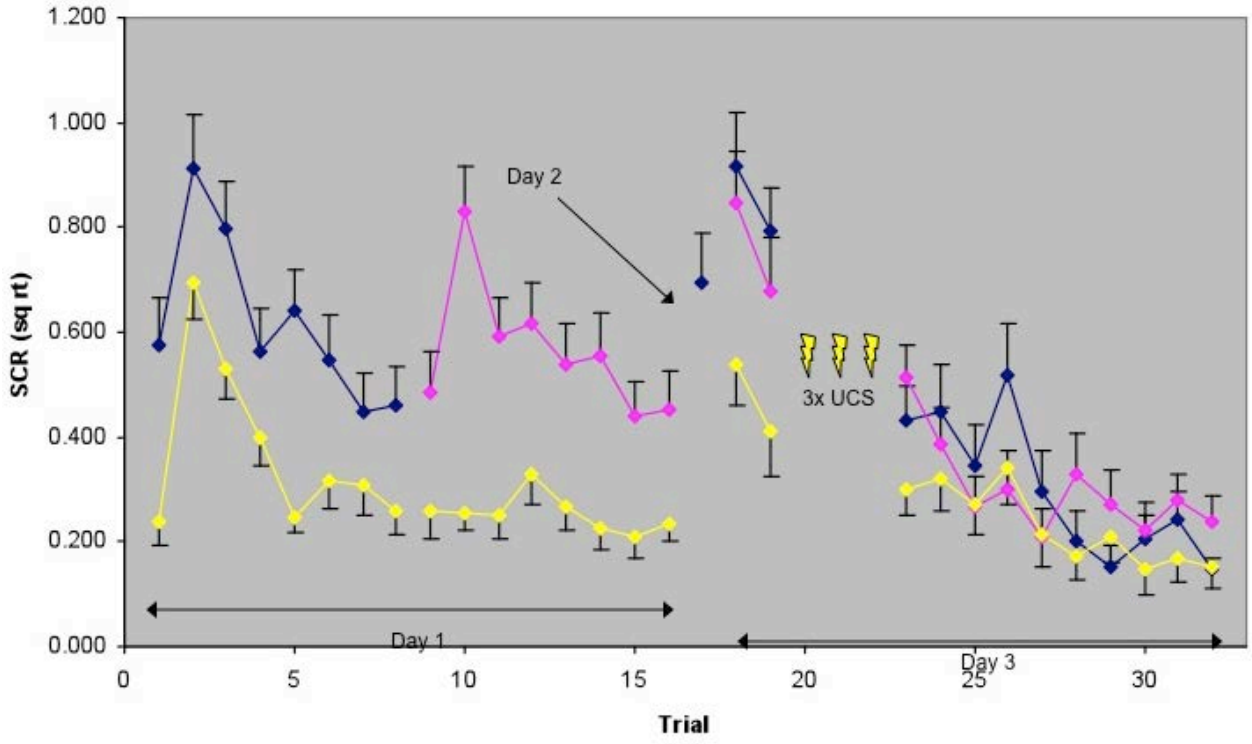
7. APPENDICES

FIGURE LEGENDS

Figure 1: This figure includes participants (n=44) with measurable skin conductance (SC) responses on Day 1. The upper panel shows averaged SC responses for the reactivated CS+ (CS+R, blue) and non-reactivated CS+ (CS+N, pink) trials versus the respective CS- (yellow) trials. Responses during the acquisition phase appear in the left of the upper panel. In the middle of the upper panel is the response to the reactivated CS+ (CS+R) on Day2. In the right portion of the upper panel are the reinstatement trials (presented prior to the unsignaled shocks) and renewal trials (following the shocks) presented on Day 3. Subjects showed good differential conditioning, but failed to show evidence of reconsolidation blockade. The results for Day 30 are depicted in the lower panel. Participants returning on day 30 for follow-up (n=26) tended to be more responsive to the initial two presentations of the CS+N, compared to the CS+R and CS- trials, suggesting a reduced fear response to the CS+R, as measured by skin conductance reactivity. However, differences in the conditioned fear response were not evident in the re-extinction and re-acquisition phases (right portion of lower panel).

Figure 2: This figure includes participants (n=18) who demonstrated strong conditioning to both, the reactivated CS+ (CS+R, blue) and non-reactivated CS+ (CS+N, pink) trials versus the respective CS- (yellow) trials. Figure 2's upper and lower panels are organized the same as for Figure 1 above. As can be see in the left side of the upper panel, subjects showed very strong differential conditioning and, similar to the full sample, failed to show evidence of reconsolidation blockade. As can be seen in the lower panel, somewhat similar to the Day 30 results for the full sample, the reduced sample of participants returning on Day 30 tended to be more responsive to the initial two presentations of the CS+N, compared to the CS+R and CS- trials, suggesting a reduced fear response to the CS+R, as measured by skin conductance reactivity. However, similar to results for the full sample, differences in the conditioned fear response were not evident in the re-extinction and re-acquisition phases (right portion of lower panel).

Figure 1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Day 1
Day 3
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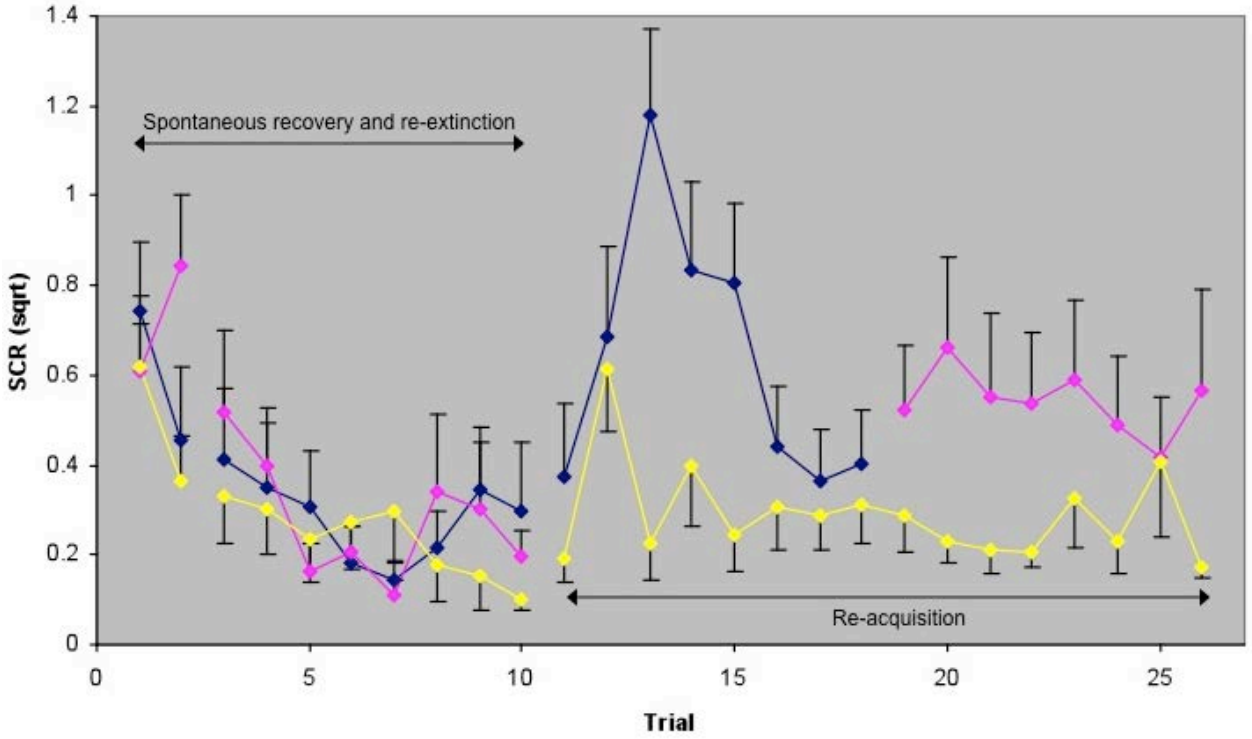
5
10
15
20
25
30
Trial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0.6
0.4
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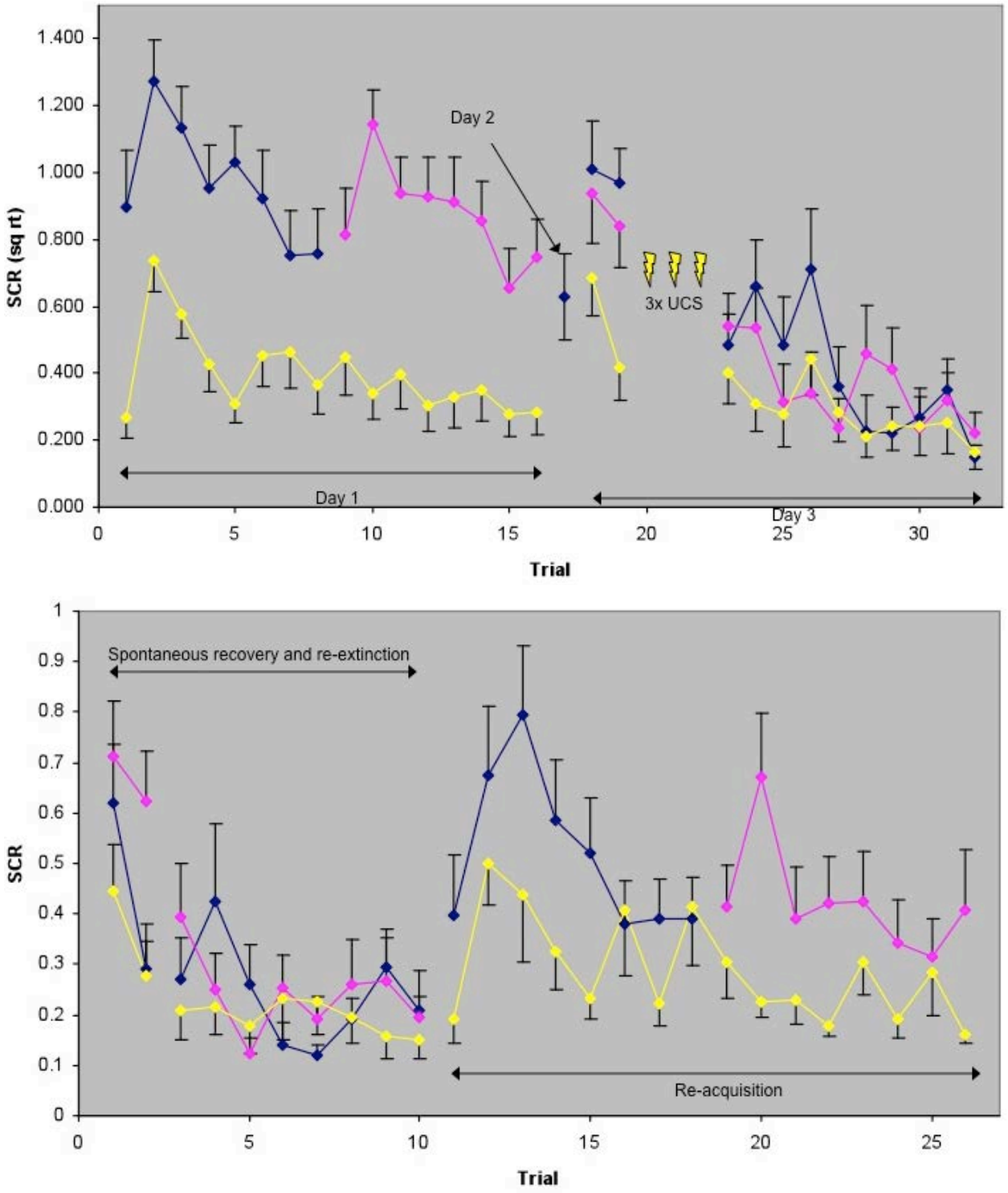
SCR (sqrt)
Spontaneous recovery and re-extinction
Re-acquisition
0
5
10
15
20
25
Trial

Figure 2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1.000
0.800
0.600
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0.200
0.000
Day 2
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Day 1
Day 3
0
5
10
15
20
25
30
Trial
SCR
1
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0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Spontaneous recovery and re-extinction
Re-acquisition
0
5
10
15
20
25
Trial